Remarks/Arguments

Summary of Disposition of the claims

Claims 31-34 are currently pending in the application and are the subject of a restriction requirement. Elected claims 35-36 are rejected and non-elected claims 31-34 are withdrawn from consideration.

Applicants respectfully request cancellation of withdrawn claims 31-34 without prejudice to Applicants' right to pursue those claims in subsequent divisional applications.

Applicants respectfully request cancellation of rejected claim 35 without prejudice.

The rejection under 35 USC §112, first paragraph

The Examiner has rejected claims 35-36 under 35 USC §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Applicants have cancelled claim 35, thus obviating the rejection of this claim. Regarding the rejection of claim 36, Applicants respectfully disagree with the Examiner for the following reasons.

Applicants disclose on page 15, lines 23-26, "Since noggin is expressed in the branchial arch neural crest, we believe it may therefore influence whether neural crest cells deposit cartilage...".

On page 16, lines 17-23, Applicants disclose, "Because noggin has a pattern of expression that suggests it is used to regulate cartilage production in the embryonic head, clinical uses to regulate cartilage and bone growth are suggested for noggin in therapeutic compositions and particularly in combination with other growth factors due to a property of noggin to potentiate at least some growth factors."

Applicants disclose on page 36, lines 16-21, "Expression of noggin initiates at several new sites, which become progressively clearer as the tadpole matures. A discontinuous line of stained cells runs the length of the roof plate of the neural tube. Staining is also apparent in the head mesoderm, primarily in the mandibular and gill arches. We suspect that this expression corresponds to skeletogenic neural crest cells."

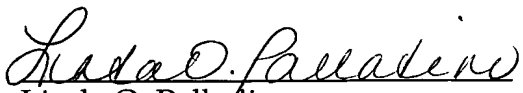
Together, these disclosures support Applicants assertion that noggin is involved in the regulation of cartilage deposit and skeletogenesis. In further support of Applicants assertions, attached herewith is Brunet, *et al.*, (1998) Noggin, Cartilage Morphogenesis, and Joint Formation in the Mammalian Skeleton, Science 280:1455-1457, which describes noggin's role in cartilage regulation and skeletogenesis. This paper provides independent

corroboration that Applicants assertions regarding noggin's role in cartilage regulation and skeletogenesis were in fact correct.

In light of the arguments set forth above and the supporting document attached herewith, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claim 36 under 35 USC §112, first paragraph.

No fee is deemed necessary in connection with filing this paper. However, if any fee is necessary, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 18-0650.

Respectfully submitted,



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ture, the partition function and the matrix of base pairing probabilities computed from the Boltzmann ensemble, and the complete set of suboptimal folds in the vicinity of the minimum free energy.
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13. The shape α itself occurs in every sequence neighborhood of the α sample (omitted from Fig. 2). This reflexivity of the nearness relation is the topological way of expressing neutrality.
14. Coarse-grained shapes are derived from secondary structures by ignoring the size of stacks and loops, keeping only their relative arrangement. Our tRNA boundary sample (see legend to Fig. 2A) contained 5882 coarse-grained shapes. A pool of 11,000 random sequences yielded 1578 distinct coarse-grained shapes, 90.4% of which were found in the tRNA boundary.
15. An example for a discontinuous transition of type i is the formation of a multiloop (a loop issuing more than two stacking regions). Generally, the free energy gain upon formation of a stack must offset the free energy loss from the loop caused by it. A stack closing a multiloop must, therefore, come into existence with some minimum length (typically more than 5 base pairs) in a single step. Likewise, the discontinuity of generalized shifts (type ii) has thermodynamic and structural origins. Shifting a stack by sequentially shifting its base pairs in random order would cause severe sterical conflicts, besides violating the formal no-knot condition. As a consequence, the shifting of a stack requires that all base pairs move synchronously.
16. Most, but not all, phenotypes on the path are highly populated. A path inferred from the fossil record almost certainly misses the low populated ones.
17. The shapes on the evolutionary path, including an "active" version of Fig. 1A, and additional information are available at www.santafe.edu/~walter/RNA/punct.html and www.tbi.univie.ac.at/~walter/RNA/punct.html.
18. The average number of replication events per time unit depends on the average replication rate constant in the population. The plateaus preceding events a and b had a duration comparable to those preceding events g and h, but during the former about 4300 replications occurred per time unit, whereas during the latter this number rose to 10,800.
19. Discontinuous transitions may trigger a cascade of continuous events. On a few occasions, continuous transitions hitchhike on discontinuous ones. For example, a major rearrangement, such as a double flip, may involve the simultaneous elongation of a stack formed in the event.
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27. Financial support was provided by the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (Projects P-10578 and P-11065), by IIASA Laxenburg, Austria, by the Commission of the European Union (Contract Study PSS*0884), and by the integrative core research at the Santa Fe Institute.

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Noggin, Cartilage Morphogenesis, and Joint Formation in the Mammalian Skeleton

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Noggin is a bone morphogenetic protein (BMP) antagonist expressed in Spemann's organizer. Murine Noggin is expressed in condensing cartilage and immature chondrocytes, as are many BMPs. In mice lacking Noggin, cartilage condensations initiated normally but developed hyperplasia, and initiation of joint development failed as measured by the expression of growth and differentiation factor–5. The maturation of cartilage and Hoxd expression were unaffected. Excess BMP activity in the absence of Noggin antagonism may enhance the recruitment of cells into cartilage, resulting in oversized growth plates; chondrocytes are also refractory to joint-inducing positional cues.

The bones of the developing limb bud are formed by condensations of chondrocytes followed by endochondral ossification. Postembryonic growth continues at the growth plates, at the ends of the bones (*1*). A series of inductive events determines the sizes and shapes of individual limb skeletal elements (*2*). Many growth factors of the BMP family have been implicated in limb growth and patterning (*3*). BMP2 and BMP4, for example, when overexpressed during chick limb development alter the size and shape of the long bones, probably by increasing recruitment of nondifferentiated limb mesenchyme into the chondrogenic pathway (*4*, *5*). The joints are formed after the initial cartilage condensation and are first recognized histologically by an increase in cell density. Cell death and cavitation follows (*1*). The location of the joints defines the layout of the skeleton. Growth and differentiation factor–5 (GDF-5), a divergent member of the BMP family, is implicated in joint specification through its expression in prospective joints and its disruption in the *brachypodism* mouse mutation (*6*).

BMP activities may be modulated not only through gene expression and protein processing, but also by interaction with antagonists such as Noggin and Chordin (*7*). Here we show that Noggin expression is essential for proper skeletal development; excess BMP activity in the Noggin null mutant resulted in excess cartilage and failure to initiate joint formation.

A null mutation was made in the mouse gene *Noggin* by replacing the coding sequence with the lacZ gene, deleting all but 10 NH_2-terminal residues of *Noggin*. The effect of this mutation on the central nervous system and somite patterning is discussed elsewhere (*8*). This null mutation should cause overactivity of BMP proteins, because the BMP antagonist, Noggin, has been removed.

Although heterozygous embryos are normal, all skeletal structures are abnormal in the homozygous mutant, with striking defects in the vertebrae, ribs, and limbs (Fig. 1). The severity of the axial defects increases caudally. The skull and cervical vertebrae are nearly normal, but the thoracic vertebrae are fused, fail to close dorsally, and do not develop a neural arch, and the lumbar and tail vertebrae are missing. The ribs are abnormally few and branched, and sternal bands fail to fuse completely (*9*). Alizarin red staining of bone shows that the schedule of ossification for both axial and limb skeleton in the homozygous mutant appears normal. Although defects in the axial skeleton suggest that Noggin affects cartilage development, the early action of Noggin in midline patterning complicates interpretation of the phenotype (*8*). Consequently, we focused our analysis on the limb.

The mutant limbs are shorter than those of the wild type and broadened along the anterior-posterior axis (Fig. 1D). Fusion of the joints is particularly evident at the elbow, where the radius and humerus are joined by a continuous ossification. The digits have secondary fusions and occasional cartilaginous spurs, and they lack joints.

We analyzed normal Noggin expression in heterozygous embryos using the lacZ transgene (Fig. 2A) (*10*). In situ hybridization (*11*) of a *Noggin* antisense probe to whole embryos or sectioned limbs con-

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firmed that lacZ staining reflects authentic *Noggin* gene expression (*9*, *12*) and correlates with the sites of abnormal development of the skeleton. Expression of Noggin in forelimb cartilage of the 16.5-dpc heterozygote is widespread but is extinguished in the developing joint cavity and in maturing hypertrophic cartilage (Fig. 2B). Expression at the articular surface persists into adult stages (*12*).

To distinguish whether joints were absent from the maturing skeleton as a consequence of excess chondrocyte growth and secondary fusions, or a failure to divide the initial condensation, we examined the onset of gene expression in the joint. Noggin (pink) and GDF-5 (blue) expression were analyzed in 11.5- to 14.5-dpc embryos (Fig. 2, C to N). The initial chondrocyte condensations of heterozygous and homozygous mutant mice are indistinguishable. At first, GDF-5 is expressed around and at the borders of the condensations. As cartilage develops, GDF-5 expression is up-regulated in the prospective joints (Fig. 2). By 13.5 dpc (Fig. 2, I to K), the mass of cartilage expressing Noggin or seen histologically (*19*) is greater in null mutant embryos (Fig. 2K) and seems to occur at the expense of the residual limb mesenchyme. At 13.5 dpc, GDF-5 expression is apparent in prospective joints of the phalanges in normal embryos (Fig. 2, I and J) but not the homozygotes, even though GDF-5 is expressed in the interdigital mesenchyme (Fig. 2K), where it may also have a role in cell death (*13*). By 14.5 dpc, much of the growth of the limb in the long axis is confined to the growth plates, and the continued failure to specify joints, especially in the digits, probably leads to the slightly shorter length of mutant limbs at birth (Fig. 1, B and D). Examination of sectioned material confirmed that, whereas GDF-5 is normally

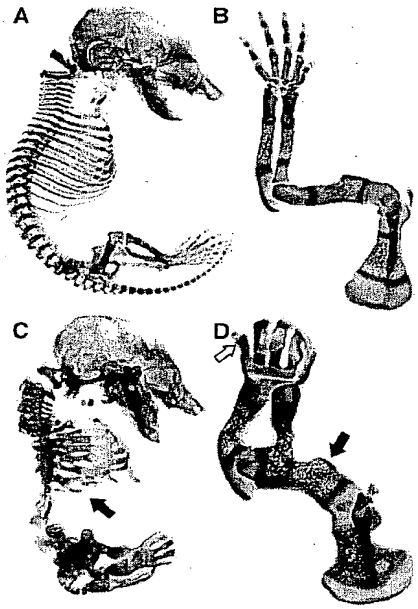

Fig. 1. Skeletal abnormalities in *Noggin* homozygous mutants. Skeletons, with forelimbs removed, from wild-type (**A**) and mutant (**C**) 18.5-dpc (days post coitum) embryos were stained with alcian blue for nonmineralized cartilage and alizarin red for mineralized cartilage and bone (*21*). The forelimbs are shown in (**B**) and (**D**), respectively. In (D), continuous ossification from the radius to humerus is indicated by the solid arrow and a cartilaginous spur by the open arrow.

Fig. 2. Expression of Noggin and GDF-5 in the developing skeleton. Heterozygous embryos at 13.5 (**A**) or 16.5 (**B**) dpc were stained to detect lacZ activity (*10*). A section of a forelimb is shown in (B): j, joint; p, proliferating zone; h, hypertrophic cartilage. In panels (C) through (N) GDF-5 expression was detected by whole-mount in situ hybridization (blue) (*11*); Noggin/lacZ expression in heterozygotes (+/–) and homozygotes (–/–) was detected with salmon-gal staining (pink) (*22*). Bars are 1 mm. All limbs are right hindlimbs with anterior at top. (**C** through **E**) 11.5 dpc; (**F** through **H**) 12.5 dpc; (**I** through **K**) 13.5 dpc; (**L** through **N**) 14.5 dpc. Wild type (+/+) (C, F, I, L); heterozygous (+/–) (D, G, J, M); homozygous (–/–) (E, H, K, N). GDF-5 transcripts are first detected in the developing phalanges at 12.5 dpc [(F) and (G)] but are not detected in the homozygous mutant limb (H). GDF-5 expression is peripheral to the sites of cartilage condensation that are revealed by Noggin expression, except where joints are to be formed (I, J, L, M). The overgrowth of cartilage in the mutant limb is seen by the extensive area of pink (lacZ) stain in (N), and is confirmed histologically (P). The arrow in (N) marks a cartilaginous spur. GDF-5 expression is shown in sectioned limbs from (**O**) wild-type and (**P**) homozygous mutant 16.5-dpc embryos (*11*).

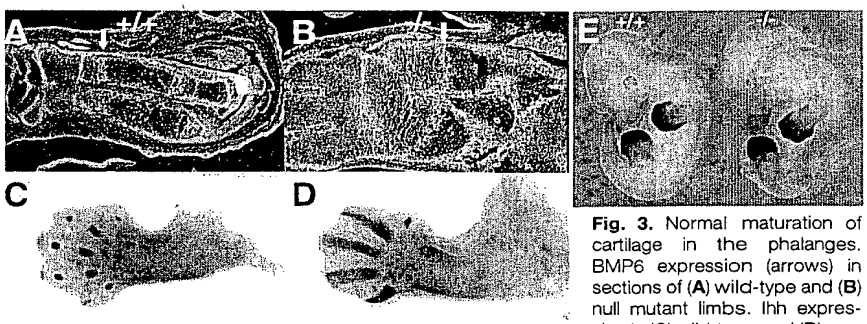

Fig. 3. Normal maturation of cartilage in the phalanges. BMP6 expression (arrows) in sections of (**A**) wild-type and (**B**) null mutant limbs. Ihh expression in (**C**) wild-type and (**D**) mutant limbs. Ihh marks maturing cartilage in the zone between prospective joints (*14*). (**E**) Hoxd13 expression at 12.5 dpc in wild-type (+/+) and mutant (–/–) embryos.

expressed in all joints at 16.5 dpc (*1*), expression is absent from similar positions in the homozygous mutant (Fig. 2, O and P).

Elevated BMP activity in the Noggin null mutant may cause misregulation of BMP-dependent genes including the BMP genes themselves, which are up-regulated in the notochord of Noggin mutant embryos (8). BMP6 is expressed in the hypertrophic zone of cartilage and in the joints (*14*) (Fig. 3A). BMP6 expression is normal in the Noggin mutant (Fig. 3B), as is expression of BMP2, BMP4, and BMP5 (*12*). Therefore, defects in the Noggin null mutant are likely to result from the lack of Noggin protein and the consequent inability to locally antagonize BMP activity, rather than from changes in the expression patterns of members of the BMP family.

Other markers of cartilage maturation, such as Indian hedgehog (Ihh; Fig. 3, C and D) (*15*) and parathyroid hormone-related protein (PTHRP) (*12*, *15*), are also expressed in Noggin mutants, albeit in an expanded cartilage field unbroken by normal discontinuities at the joints. Thus, ossification proceeds on schedule (Fig. 1). A similar extended expression of Ihh occurs in chick embryos with elevated BMP concentrations (*5*). There, the failure of digital joint formation may also produce a single extended cartilage element, which matures synchronously.

Because failure of joint formation in the Noggin null mutant is correlated with a failure to up-regulate GDF-5 expression in the presumptive joint regions, the absence of joints in the mature limb cannot be a consequence of the secondary fusion of condensations, but must result from the failure to specify the joint. Only a subset of joints is affected in the *brachypodism* mouse, which has a mutation in the gene encoding GDF-5 (*1*, *6*), suggesting that additional factors are involved in joint initiation.

A possible cause of the failure to specify joints in the mutant limb might be the loss of positional information that would normally specify where GDF-5 expression should initiate. To date there is no information on the upstream regulation of GDF-5, but changes in joints and skeletal elements also result from mutations in genes encoding Hox proteins (*16*). Elevated BMP activity in the Noggin null mutant could cause changes in Hox expression patterns (*17*, *18*). However, at the time when joints in the phalanges are being specified, Hoxd13 and Hoxd11 expression is unaffected in the Noggin mutant, indicating that the positional information is unaltered (Fig. 3E) (*9*, *12*).

The limb phenotype of the Noggin null mutant is similar to the effects resulting from overexpression of BMPs in the developing chick limb (*4*, *5*). BMP4 and BMP7 are expressed in the perichondrium (*4*, *5*), which is a source of stem cells for appositional growth as well as a barrier to cartilage expansion (*19*). Application of excessive BMP2 or BMP7 to the developing chick limb bud before mesenchyme condensation induced apoptosis (*5*) and a subsequent loss of skeletal elements. Differentiating chondrogenic cells were unaffected. If the BMPs were applied later, the skeleton resembled the abnormal skeleton of the mouse Noggin null mutant. Thus, we conclude that Noggin acts to regulate BMP activity after chondrogenesis has initiated. The increased BMP activity increases the recruitment of cells into cartilage, expanding the cartilage at the expense of other tissues and causing larger growth plates (*9*).

Joint identity may require a particular balance of BMP activities to make chondrocytes competent for joint formation. Currently the best candidates for regulators of expression of GDF-5 and other BMPs are members of the HoxA and HoxD complexes (*2*, *20*). Mutations in the genes encoding these proteins cause limb defects, and the phenotypes, which can include growth defects and an absence of particular elements, have been interpreted to result from changes in the rates of cell proliferation or condensation or both (*17*). In the absence of Noggin, and the consequent absence of regulated BMP activity, we propose that chondrocytes are unable to respond to instructions from the Hox complex, leading to a failure to turn on genes like the one encoding GDF-5. Thus, it is apparent that a balanced combination of positive and negative factors is required to define and shape each skeletal element.

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22. For β-galactosidase staining, embryos were stained for lacZ activity as described (*10*). For Fig. 3, salmongal (Biosynth International, Skokie, IL) was substituted for X-Gal. Embryos were fixed in 4% paraformaldehyde and processed for whole-mount in situ hybridization (*11*).

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